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(54) Transformation of trichoderma.

(57) A vector system for transformation of *Trichoderma* has been developed. The vector system can be used for high expression and secretion of proteins in *Trichoderma* by transformation of a suitable *Trichoderma* strain with the vector system comprising a gene for a desired protein.

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The present invention relates to a vector system for use in the transformation of Trichoderma, a process for high level expression and secretion of proteins in Trichoderma, DNA recombinant vectors and transformed Trichoderma strains.

BACKGROUND OF THE INVENTION

Filamentous fungi are lower eukaryotes widely used in biotechnology to make various fermentation products. Fungi secrete many industrially important enzymes such as glucoamylase, proteases, lactase, pectinases and glucose oxidase.

Filamentous fungi have a number of biotechnical advantages. They generally produce high amounts of proteins, cultivation in large scale is not complicated and separation of the mycelium from the culture liquid after the fermentation is easy.

The mesophilic imperfect fungus Trichoderma reesei (formerly T. viride) (ref. 1) produces enzymes needed in conversion of cellulosic biomass and is probably the most widely investigated of all cellulase-producing organisms.

For hydrolysis of cellulose to glucose, three types of enzyme activity are needed: randomly cleaving endoglucanases (1,4-β-D-glucan glucanohydrolase, EC 3.2.1.4) which usually attack substituted soluble substrates and show no activity to crystalline cellulose; cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91) capable of degrading crystalline cellulose but having no activity towards derivatized cellulose and β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) attacking cellobiose and cello-oligosaccharides to yield glucose. Synergistic action between some of these enzymes has been demonstrated (refs. 2-4).

Fungal cellulases have been purified and characterized and all three main types of enzymes have been shown to occur in multiple forms (ref. 5). Two immunologically distinctive cellobiohydrolases, CBH I and CBH II have been detected from the culture medium of T. reesei (refs. 4, 6). Five to eight electrophoretically distinct endoglucanases have been reported, many of them showing varying substrate specificities (refs. 7, 8, 9, 10). Characterization of two extracellular β-glucosidases has been reported (ref. 11).

Intensive strain development using the direct approach of mutation and screening has successfully produced several high-yielding T. reesei mutant strains (refs. 12-22). The amount of extracellular protein produced by the best T. reesei mutants is as much as 20-30 g/litre of culture fluid which is more than 50 % of the total cell protein. A major part of the secreted protein comprises cellulases among which the CBH I component is most abundant, representing up to 60 % of the secreted cellulase proteins.

The gene for CBH I has been cloned by Shoemaker et al. (ref. 23) and Teeri et al. (ref. 24) and the whole nucleotide sequence of the gene has been published (ref. 23). From T. reesei, also the gene for the major endoglucanase EG I has been cloned and characterized (ref. 25, 26, 27). Other isolated cellulase genes are cbh2 (ref. 28, 29) and egl3 (ref. 30).

The molecular biology of industrially important filamentous fungi is in general not well known. This is partly due to the lack of the sexual reproduction cycle and/or genetical transformation system. Recently, transformation systems have been developed for Neurospora crassa (ref. 31), A. nidulans (refs. 32, 33, 34) and A. niger (ref. 35) generally having their basis in complementation of the mutant host by respective functional gene carried by the vector molecule. However, of these fungi only A. niger is of industrial interest at the moment.

In the classification of fungi, the genus Aspergillus is included in the class Ascomycetes, sub-class Eusscomycetes. Euascomycetes are divided into three groups, Plectomycetes, Pyrenomycetes and Discomycetes on the basis of the fruiting bodies. The most important genera are Aspergillus and Penicillium (ref. 56). Trichoderma, instead, is classified as a member of Fungi imperfecti. Fungi imperfecti is a catch-all cathegory of fungi which have no sexual reproduction or obvious affinities with sexually reproducing genera, such as the highly characteristic Aspergillus. Although Trichoderma has been reported to possess a poorly defined sexual stage being an imperfect state of the perfect ascomycete species Hypocrea (ref. 57), the genera Aspergillus and Trichoderma are clearly to be considered taxonomically very different. It has also been shown that the argb gene from Aspergillus nidulans and the pyr4 gene from Neurospora crassa do not hybridize, under non-stringent conditions, to the respective Trichoderma genes thus supporting the idea that Trichoderma is evolutionary quite distinct from other Ascomycetes (Vananen, S., in preparation).

Due to its exceptional ability to secrete proteins into the growth medium Trichoderma reesei is a good candidate as a possible host for the production of proteins. However, genetic studies of T. reesei have so far been directed almost exclusively to improve the cellulase-producing properties of the fungus and the only technique used for the development of hypercellulotic Trichoderma strains has been traditional mutagenesis and screening. No transformation systems for Trichoderma have so far been developed.

It is the main object of the present invention to provide an effective host-vector system for Trichoderma reesei to obtain a high level expression and secretion of heterologous proteins in Trichoderma or to enhance the production of homologous proteins.

In the present specification with claims, the expression "proteins heterologous to Trichoderma" means proteins not naturally produced by Trichoderma whereas "proteins homologous to Trichoderma" means proteins produced by Trichoderma itself.

In the fungal transformation systems first developed, namely for Aspergillus and Neurospora, transformation is carried out using protoplasts. The transforming DNA is usually integrated into the host genome. To provide a selection system for identifying stable transformants the vector system must carry a functional gene (a selection marker) which either complements a corresponding mutation of the host genome or supplies an activity, usually an enzyme, required for the growth of the prototrophic strain on a particular growth medium.

SUMMARY OF THE INVENTION

The present invention describes a recombinant DNA cloning vector system for the transformation of Trichoderma. When the DNA cloning vector system comprises a DNA-sequence encoding a desired protein product transformation of Trichoderma with the present vector system will provide a high level expression and secretion of the desired protein when the transformed microorganism is grown in a suitable culture medium.

According to its first aspect the present invention provides a vector-system comprising

a) a gene encoding a desired protein product,

b) function facilitating gene expression including promoters/enhancers operably linked to control expression of the desired product,

c) optionally a signal/leader sequence fused upstream to 5' end of the gene for the desired product and

d) a selection marker.

According to its second aspect the present invention provides a method for transformation of Trichoderma, wherein a suitable Trichoderma strain is transformed with the present vector system.

According to its third aspect the present invention provides a method for the production of a protein in Trichoderma which comprises transforming Trichoderma with the present vector system, culturing the transformed strain in a suitable medium and recovering the expressed and secreted product from the medium.

The present invention also provides a method for the production of a protein in Trichoderma by which method a Trichoderma strain transformed with the vector system is cultivated in a suitable culture medium and the expressed and secreted protein is recovered from the culture medium.

The present invention furthermore provides stably transformed Trichoderma strains.

As used herein the expression "vector system" includes a single vector or plasmid or two or more vectors or plasmids which together contain the DNA-information to be integrated into the host genome. The vectors or plasmids may be linear or closed circular molecules. Although self-replicating plasmids in Trichoderma are not known at present the invention also covers such self-replicating plasmids if they should be found at a later time.

The vector system comprises DNA-sequences encoding functions facilitating gene expression including promoters, enhancers and transcription initiation sites as well as terminators, a marker for selection of transformants and a DNA-sequence encoding the desired protein.

To ensure secretion of the expressed product the gene for the desired product is preferably provided with a preregion ensuring effective direction of the expressed product into the secretory pathway of the cell. This preregion which might be a naturally occuring signal or leader peptide or parts thereof is generally cleaved off from the desired product during

secretion whereupon the mature product can be isolated from the culture medium.

The gene for the desired product may be obtained from genomic clones or cDNA clones. DNA-sequences may also be synthesized.

The signal/leader sequence may be derived from the signal/leader sequence of the gene encoding the desired protein or may be derived from genes for other secreted proteins either from Trichoderma or from any other source of organism. Examples of signal/leader sequences derived from genes encoding a protein secreted by Trichoderma are the cbh1 signal sequence or the egl1 signal sequence or parts thereof. Signal/leader sequences derived from genes encoding secreted proteins heterologous to Trichoderma may be derived from genes for Aspergillus amylases or glucoamylase. Also synthetic signal/leader sequences may be used.

The promoter may be any DNA-sequence that shows transcriptional activity in Trichoderma and may be derived from genes either homologous or heterologous to Trichoderma. Examples of promoters derived from genes encoding proteins homologous to Trichoderma are the cbh1 promoter or the egl1 promoter or parts thereof. An example of a promoter derived from a gene for a protein heterologous to Trichoderma is the Aspergillus glucoamylase promoter. Transcription terminators may be derived from the same sources as the promoters. Also synthetic promoter or terminator sequences may be used.

It is understood and obvious to the expert in the art that all specifically mentioned DNA-sequences may be modified by amendment or deletion of a couple of bases non-essential to the function of the product encoded for. For example DNA sequences substantially similar to cbh1 or egl1 signal or promoter sequences may be used as long as they exhibit the intended function in Trichoderma. Also the genes for the desired proteins may be altered as long as this has no deleterious effect on the activity of the protein.

Different selection markers may be used, e.g. argB (A. nidulans or T. reesei), amdS (A. nidulans) and pyr4 (Neurospora crassa or T. reesei).

The host strain may either be a prototrophic or an auxotrophic Trichoderma strain depending on the selection marker used in the transformation procedure. The amdS gene from A. nidulans may as demonstrated in the experimental part of the specification be used for the transformation of prototrophic T. reesei strains. T. reesei grows poorly on

acetamide as the sole nitrogen source and the growth can be further inhibited by adding CsCl to the medium. The growth on acetamide as the sole nitrogen source in the presence of CsCl can accordingly be used as a selection medium to identify $Amd8^+$ transformants.

If a selection marker is used which complements a corresponding mutation of the host genome auxotrophic Trichoderma mutants must be constructed. Auxotrophic Trichoderma mutants may be produced by known methods for producing mutant strains.

Auxotrophic Trichoderma mutants requiring uracil, tryptophan or arginine for growth were isolated by a filtration enrichment technique as described by Nevalainen (ref. 36). From arginine requiring auxotrophs, mutants deficient in the argB gene were identified by using a series of minimal plates supplied by different intermediates in the arginine biosynthesis. From uracil-requiring mutants, strains deficient in the pyr4 gene can be sought for by measuring the orotidine-5'-phosphate decarboxylase (OMP decase) activity in mycelium preparations (ref. 37).

Mutants having a trp1 gene defect can be characterized by the lack of the PRA isomerase - InGP synthetase activity in their mycelia (ref. 40). The trp1 -character of the mutants showing no enzyme activity may be confirmed by e.g. transformation and complementation with the N. crassa trp1 plasmid (ref. 41) or the trpC -plasmid of A. nidulans (ref. 34).

The transformation technique used is the method adapted from the methods for transformation of A. nidulans (refs. 32, 33). Protoplasts are prepared by known methods by growing mycelium on agar plates and suspending mycelium in a buffered solution of Novozym^R 234. Instead of the conventional sucrose solution of Novozym^R 234 a sorbitol solution may advantageously be used. Transforming DNA is then added to the protoplast solution as described in further detail in the experimental part of the specification. The transformation is usually carried out as a cotransformation by which a nonselectable plasmid is cotransformed into T. reesei with high frequency using a selectable marker inserted in another plasmid (e.g. amdS in p3SR2 or argB in pSal43). A great proportion of the transformants contain the nonselectable plasmid integrated into the genome if equimolar amounts of DNA are used for transformation. When an argB$^-$ T. reesei strain is used in transformation with equimolar amounts of plasmids p3SR2 and pSal43, transformants can be obtained on double selection medium (minimal medium, acetamide, CsCl). Alternatively a single plasmid may be used for transformation in which also the selectable marker is inserted.

Transformants are then cultured in a suitable medium. To be able to produce the desired product in a simple defined medium a glucose inducible promoter may be used. When Trichoderma is grown on a medium containing 1 % glucose, secretion of all extracellular proteins, including proteases is strongly suppressed. When the gene coding for the desired enzyme is connected to a promoter strongly expressed in glucose containing medium, the desired enzyme is secreted to the growth medium. Since other proteins are not produced under these conditions, the desired enzyme is the major protein secreted into the medium.

When the fungal mycelium is removed the desired protein is present in the resulting solution in a very pure form. If required, it can be very easily further purified, since it is almost the only protein present.

The present invention may also be used to modify or inactivate a gene producing a certain enzyme activity of host organism. As an example cellulase-negative T. reesei strains can be constructed. This mutagenesis is based on transformation of Trichoderma reesei with a plasmid carrying a defected cellulase gene. Homologous recombination of the plasmid at the chromosomal cellulase locus causes insertional inactivation of the endogenous T. reesei cellulase gene. The plasmid used for transformation contains only part of the cellulase coding region and produces inactive protein. No 5' flanking sequences are included. A frameshift mutation can also be introduced to the truncated coding region. A selection marker (argB) or a marker for screening (lacZ) can be coupled to the plasmid used for transformation. After recombination the marker will be placed between the two resulting defective cellulase genes. Principle of the method is shown in fig. 1.

The present invention is illustrated by means of the production of chymosin and Trichoderma reesei endoglucanase I (EGI). Promoter, signal and leader and terminator sequences were derived from either the glucoamylase gene from Aspergillus niger or the Trichoderma reesei cbhl gene. As selection marker the amdS gene from A. nidulans was used.

Materials

Plasmids:

p285:	ATCC No. 20681
pCAMG91:	ref. 59
piC19R:	ref. 60
pSa143:	refs. 51 + 54
p3SR2:	refs. 33 + 35
pDJB2:	ref. 38
pMC1817:	ref. 46
pTT01, pTT11:	ref. 28
pUC9, pUC13:	ref. 61
pUC18, pUC19:	ref. 58
pTZ19R:	(Pharmacia)
pAN5-41B:	ref. 62

E. coli strains JM101, JM103, JM109 and DH1 (ref. 59) are used as hosts in E. coli cloning. Trichoderma reesei strains QM9414 (ATCC 26921) and RUT-C-30 (ATCC 56765) are used in fungal transformation and expression studies.

Trichoderma minimal medium:

Glucose	20 g
$(NH_4)_2SO_4$	5 g
KH_2PO_4	15 g
$MgSO_4$	0.6 g
CaCl	0.6 g
$FeSO_4, 7H_2O$	5 mg
$MnSO_4, H_2O$	1.56 mg
$ZnSO_4, 7H_2O$	1.4 mg
$CoCl_2$	2 mg / 1 H_2O

Brief description of drawings

The figures of the constructions are not in scale.

Fig. 1 shows the principle of the inactivation of a chromosomal cellulase gene.

Fig. 2 shows the construction of plasmids pMS4 used for insertion mutagenesis of the T. reesei chromosomal CBHI locus. Position of a frameshift mutation generated by inactivation of EcoRI site is marked with *.

Fig. 3 shows the construction of plasmid p285' proC.

Fig. 4 shows the construction of plasmids pMT648 and 15 pMT813.

Fig. 5 shows the construction of plasmid pMT837.

Fig. 6 shows the restriction map of plasmid pR27.

Fig. 7 shows the restriction map of plasmid pAMH100.

Fig. 8 shows the construction of plasmid pAMH105.

Fig. 9 shows the construction of plasmids pAMH1103, pAMH1106 and pAMH1101 by loop-mutagenesis using synthesized oligonucleotides OAMH1, OAMH2 and OAMH3.

Fig. 10 shows the linkers NOR 202, NOR 203, OAMH1, OAMH2 and OAMH3.

Fig. 11 shows the restriction maps of plasmids pAMH102 and pAMH104.

Fig. 12 shows the restriction map of plasmid pAMH1103 and the construction of pAMH103.

Fig. 13 shows the restriction map of plasmid pAMH1106 and the construction of pAMH106.

Fig. 14 shows the restriction map of plasmid pAMH1101 and the consruction of pAMH101.

Fig. 15 shows the construction of plasmid pAMH110.

Fig. 16 shows the construction of plasmid pAMH111 used for expressionof EGI under CBHI promoter function.

Fig. 17 shows the expression of chymosin in T. reesei, Western blot. Lane 1; purified prochymosin control, includes traces of pseudochymosin and chymosin. Lane 2; culture supernatant from growth of strain including pAMH102. Lane 3; control supernatant from strain without plasmids. Lane 4; mycelia from strain including pAMH102.

Example 1

Induction, enrichment and isolation of auxotrophic T. reesei mutant strains.

Auxotrophic mutants requiring uracil, tryptophan or arginine for growth were isolated using filtration enrichment as described by Nevalainen (ref. 36). The mutagenic agent used was UV-light. From arginine requiring auxotrophs, mutants deficient in the argB gene were identified by using a series of minimal plates supplied by different intermediates in the arginine biosynthesis (ref. 36). Mutants requiring citrulline for growth were considered as possible argB$^-$ mutants. The argB$^-$ character of isolated mutants was confirmed by transforming them into prototrophy using the plasmid pSal43 containing the A. nidulans argB gene (example 4).

From uracil-requiring mutants, strains deficient in the pyr4 gene were sought for. The pyr4$^-$ character of these mutants was confirmed by transformation with a plasmid containing the N. crassa pyr4 gene (ref. 38) (example 4).

Example 2

Preparation of protoplasts

Mycelium was grown on cellophane disks on Potato Dextrose Agar plates (Difco). Mycelium from 5 cellophane cultures was suspended in 15 ml of a 5 mg/ml solution of Novozym^R 234 in 1.2 M sorbitol buffered at pH 5.6 with 0.1 M potassium-phosphate. The mixture was incubated 1.5 - 2 h at 30°C. Protoplasts were separated from mycelial debris by filtration through sintered glass (porosity 1) pelleted for 5 min at 4000 g and washed twice with 1.2 M sorbitol - 10 mM Tris, HCl pH 7.5.

Better purification of protoplasts can be achieved by using a method described by Tilburn et al. (ref. 33). 5 mg/ml solution of Novozym^R 234 in 1.2 M $MgSO_4$ buffered at pH 5.8 was used for protoplasting. After incubation and filtration the protoplast suspension was centrifugated at 4000 g for 15 min with an overlay of an equal volume of 0.6 M sorbitol - 100 mM Tris, HCl, pH 7.0. Protoplasts formed a sharp band halfway the tube. The protoplasts were suspended in 1 vol of 1.2 M sorbitol - 10 mM Tris, HCl, pH 7.5, spun down and washed twice in 1.2 M sorbitol - 10 mM Tris, HCl, pH 7.5. The protoplasts were suspended in 1.2 M sorbitol -

10 mM $CaCl_2$ - 10 mM Tris, HCl, pH 7.5 at a concentration of 5 x 10^6 - 5 x 10^7 protoplasts/ml.

The viability of the protoplasts was estimated on Trichoderma minimal medium with 1 M sorbitol as osmotic stabilizer. The protoplasts were plated in 3 % agar overlay. The regeneration frequency was 40 - 80 % for the strain QM 9414 (ATCC 26921).

Example 3

Transformation of T. reesei using A. nidulans acetamidase (amdS) gene.

Plasmid p3SR2 was used in transformation. It contained A. nidulans DNA carrying the whole acetamidase structural gene amdS and its regulatory region amdI (ref. 45 and 33).

20 µl (4 - 10 µg) of transforming DNA was added to 200 µl of protoplast solution. 50 µl of 25 % PEG 6000 - 50 mM $CaCl_2$ - 10 mM Tris, HCl, pH 7.5 was added and the mixture was incubated for 20 min on ice. 2 ml of the PEG-solution was added and the mixture was further incubated 5 min at room temperature. 4 ml of 1.2 M sorbitol - 10 mM $CaCl_2$ - 10 mM Tris, HCl, pH 7.5 was added and the protoplasts were plated in 3 % agar overlay. The selective medium was Trichoderma minimal medium with 10 mM acetamide as the sole nitrogen source instead of $(NH_4)_2SO_4$, and supplemented with 1 M sorbitol as osmotic stabilizer and 12.5 mM CsCl to repress the background growth.

Transformation frequencies from 40 to 600 transformants per µg DNA were obtained for the strain QM 9414 (ATCC 26921). The highest frequencies were obtained when the DNA was purified with two cycles of CsCl/ethidium-bromide centrifugation.

Sporulation was rarely observed on the selective medium but the transformants sporulate normally when transferred to Potato Dextrose Agar. Their ability to grow on acetamide varied. The diameter of the colonies on the selective medium ranged 1 mm to 10 - 20 mm.

The mitotic stability of the transformants was investigated. Ten large transformants of varying size were subcultured on acetamide-CsCl plates, sporulated on potato dextrose (PD) and replated on PD. To exclude heterogeneity caused by heterokaryosis, individual colonies arising from one spore were tested for $AmdS^+$ phenotype. One positive clone from each original transformant was subjected to successive platings (5 growth cycles) on non-selective medium and phenotype was tested after each

generation on the selective medium. Of 10 large transformants tested, after one cycle on non-selective medium six of the ten transformants gave 100 % AmdS$^+$ conidia, three 47-87 % and one transformant gave no AmdS$^+$ conidia. This result remained the same through five non-selective growth cycles, suggesting that the initially unstable AmdS$^+$ phenotype can be stabilized later on.

From ten original small colonies, three gave spores with variable frequencies of AmdS$^+$ phenotype (94 %, 44 % and 5 % of the spores). The other seven clones gave only spores unable to grow on acetamide. Interestingly, all AmdS$^+$ spores obtained showed vigorous growth on acetamide plates, characteristic of large colony transformants.

The presence of the plasmid DNA in the transformants was analyzed by Southern blots of total DNA, isolated from transformants (according to Raeder and Broda, ref. 44), cut with Xho I or Sal I and Eco RI. The vector pUC 18 and the Sal I - Eco RI fragment containing the amdS gene of plasmid p3SR2 were used as probes. The transformation was shown to have occurred by recombination at a number of different sites in the Trichoderma genome DNA, one to several copies per genome.

Example 4

Transformation of T. reesei with A. nidulans and N. crassa plasmids.

Complementation of auxotrophic mutations in T. reesei by heterologous DNA was demonstrated. The plasmid pSal43 containing the argB gene from A. nidulans was used to transform T. reesei argB$^-$ mutant strains. The plasmid pDJB2, containing the pyr4 gene from N. crassa was used to transform T. reesei pyr4$^-$ mutant strains. The transformants were selected on minimal medium without arginine or 30 µg/ml uracil, respectively. The frequency of transformation was in both cases around 300 (150 - 400) transformants/µg DNA.

Chromosomal DNA was isolated from the transformants and used for Southern hybridization experiments to verify the proper integration of plasmid DNA into the chromosomal DNA of T. reesei. Multiple tandem copies of pSal143 were integrated in the genome. Southern and dot blot hybridizations showed that in transformants analyzed the copy number of argB varies from appr. 2 to over 100. The ArgB$^+$ transformants were shown to be phenotypically 100 % stable through at least 3 generations. This was tested by successive platings of conidia from five transformants onto complete medium and thereafter testing the Arg$^+$ phenotype on minimal medium (50-80 colonies/transformant).

Example 5

Cotransformation of T. reesei with amdS and argB plasmids.

The possibility to cotransform Trichoderma with a non-selectable plasmid was first shown with the arginine auxotrophic mutant.

The argB$^-$ T. reesei strain was transformed with equal molar amounts of A. nidulans plasmids pSal43 (argB) and p3SR2 (amdS), transformants were selected for Arg$^+$ phenotype and tested for acquisition of amdS by streaking on acetamide-CsCl-plates. Of the ArgB transformants, 86 % were also AmdS$^+$. Southern analysis of cotransformants indicated that both plasmids were integrated as variable amounts of copies in several different locations in the genome (data not shown).

Double selection on minimal acetamide-CsCl-medium resulted in ~100 big Amd$^+$Arg$^+$ transformants per µg of DNA and a number of small colonies, characteristic of amdS transformation.

When the stability of the ArgB$^+$ phenotype of the argB amdS cotransformants was tested, three out of five proved 100 % stable, whereas the other had lost the ArgB$^+$ character in 7 % or 80 % of the progeny analysed. The same individual colonies had also lost the AmdS$^+$ phenotype. Weather this instability was caused by e.g. the cointegration of argB with the amdS to the same chromosomal location is not known.

The plasmid pAN5-4IB which contains the E. coli lacZ gene coupled in phase to the promoter and N-terminal protein coding region of the A. nidulans glyceraldehydephosphate dehydrogenase gene (gpd) (ref. 62) was also used for cotransformation. In addition, this plasmid contains the A nidulans argB gene and pBR322 sequencies. Prototrophic T. reesei (QM 9414) was cotransformed with the plasmid p3SR2 (amdS) and pAN5-4IB, transformants were selected for Amd$^+$ phenotype and screened for β-gal expression on acetamide-CsCl-plates containing Xgal. No endogenous T. reesei β-galactosidase activity could be detected when glucose was present in the medium and pH of the Xgal plates was neutral.

After 1-4 days of growth blue colour was visible. When 1.0/0.7 molar ratio of the plasmids (p3SR2/pAN5-4IB) was used in transformation 13 % of the big and 6 % of the small Amd$^+$ clones showed β-galactosidase activity, with molar ratio of 1.0/2.6, 39 % and 7 % of Xgal$^+$ clones, respectively, were obtained. The presence of both plasmids in Amd$^+$ transformants showing blue colour was verified by Southern hybridization (data not shown).

Example 6

Construction of a cbh1⁻ Trichoderma reesei ⁻strain. The activation of different cellulase genes in the manner described allows the construction of a series of T. reesei strains producing a particular combination of cellulases. cbh1⁻ strains are also important for the efficient production of heterologous proteins when using the cbh1 promoter. Trichoderma reesei strain QM 9414 (ATCC 26921) was shown to contain only one chromosomal copy of cbh1 gene by Southern hybridization using cbh1 specific probes and so one recombination event should inactivate the gene. An inactive gene was constructed as follows. The plasmid pTTO1 (ref. 27) containing the full length cDNA clone of the cbh1 gene in the vector pUC8 was cut with restriction enzymes Bgl I and Bgl II. The 0.8 kb fragment from the 5' terminal region of the cbh1 gene was isolated from agarose gel by conventional techniques. The fragment was made blunt⁻ended using S1 nuclease and it was ligated to an Eco RI cut, blunt⁻ended pUC 18 vector and transformed to E. coli JM 109. DNA from the clone containing the cbh1 gene fragment was isolated and digested with restriction enzyme Eco RI which cuts in the middle of the Bgl I ⁻ Bgl II fragment of cbh1. The Eco RI ⁻generated termini were filled in and back⁻ ligated. A plasmid pMS4 containing a frameshift mutation in the middle of the truncated cbh1 gene fragment was generated (fig. 2).

Trichoderma was cotransformed with the plasmid pMS4 and the A. nidulans amdS containing plasmid p3SR2 with 3⁻4 times molar excess of plasmid pMS4.

Transformants were selected on the basis of the amdS⁺ phenotype as described (example 3) and purified on selective medium containing asetamide as a sole carbon source. Purified transformants were grown on microtiter plates in 200 µl of Trichoderma minimal medium with 1 % Solka floc cellulose as carbon source and 0.2 % proteose peptone as nitrogen source. The cellulase phenotype was tested by the Ouchterlony immunodiffusion by using undiluted growth media against the CBH I specific antiserum (sheep) as described (ref. 59). A number of transformants were identified which produced normal amount of CBH II but no detectable CBH I.

Example 7

I. Preparation of Plasmid 285'proC Containing the Prochymosin Gene (fig. 3).

As an example of a heterologous protein in T. reesei we chose to express the prochymosin cDNA (fig. 3). The preprochymosine gene was isolated from a calf stomach cDNA library and inserted into the Pst I site of pBR322 by G-C tailing (refs. 49 and 50) to obtain pR26. pUC9 was cut with SalI and filled out with Klenow polymerase and ligated with T4 ligase. The obtained plasmid was cut with BamHI-EcoRI and the 2.7 kb large fragment was ligated with a 0.47 kb BamHI-EcoRI fragment from pR26 to create pUC9' containing a HindIII site N-terminally of the prochymosin gene. pUC13 was cut with BamHI-NarI and NarI-XmaI and the large respective small fragment was ligated with a 0.64 kb XmaI-BclI fragment of pR26 to obtain plasmid pUC13' containing an XbaI-site C-terminally of the prochymosin gene. A 0.65 kb XmaI-XbaI fragment of pUC13' was ligated with a 0.46 kb HindIII-XmaI fragment of pUC9' and a 11 kb XbaI-HindIII fragment of p285 to create plasmid p285' proC containing the prochymosin gene as illustrated in fig. 3.

II. Construction of Plasmid pAMG/Term.

pCAMG91 was digested with SalI and PstI restriction endonucleases. From such a digests a 698 bp fragment was isolated on an agarose gel. This SalI-PstI fragment contains the region encoding the 140 bp 3' untranslated part of the glucoamylase mRNA plus 540 bp 3' to the poly(A)-addition site. This 3'fragment was treated with T4-DNA polymerase to "blunt end" the restriction sites before the addition of XbaI linkers and digestion with XbaI restriction enzyme. This 3' end of the glucoamylase gene was ligated to pUC13 linearized with XbaI to create plasmid mAMG/Term containing the glucoamylase gene poly(A) addition region.

III. Construction of Plasmid pMT837

A 0.5 kb SmaII-BssHII fragment of pCAMG91 containing the promoter and the sequences coding for the glucoamylase (AMG) signal and leader peptide was ligated to SmaI-BamHI digested pUC13 and a synthetic BssHII-BamHI adaptor encoding the first 6 amino acids of prochymosin. From the resulting plasmid, pMT626, a 0.8 kb NdeI-BamHI fragment was isolated and ligated to a 0.5 kb BamHI-EcoRI fragment from p285'proC containing the

sequence for the N-terminal half of proC and to a 3.0 kb EcoRI-NdeI fragment of pMT622 containing the C-terminal part of the sequence for proC. (pMT622 is simply a EcoRI-XbaI subclone of p285' proC in pUC13). The resulting plasmid pMT648 (see fig. 4) contains the entire prochymosin gene preceeded by AMG promoter and signal/leader encoding sequences. pMT648 was further modified to contain the argB gene of A. nidulans (ref. 51). A 1.8 kb NarI-filled in XbaI fragment of pMT648 was ligated to a 6.8 kb ClaI filled-in EcoRI fragment of pSal43, to give pMT651. Sequences further upstream of the AMG promoter (upstream activating sequences; UAS's) were furthermore inserted. This was achieved by ligating a 3.7 kb ClaI-BssHII fragment from the original clone pCAMG91 to the 1.1 kb BssHII filled in XbaI fragment of pMT648 containing the proC sequences, and the argB gene as a 3.1 kb ClaI-filled in EcoRI fragment from pMT813 (pMT813 is the argB gene cloned as a 3.1 kb BamHI-NruI fragment cloned into EcoRV-BglII cut piC19R). The resulting plasmid, pMT830, has got an expression unit containing the UAS's, promoter, signal and leader sequences from AMG and the entire gene for proC. Finally the terminator sequence of AMG is taken as a 0.6 kb XbaI-XbaI fragment from plasmid pAMG/Term and inserted into the XbaI cut and dephosphorylated pMT830 to give pMT837. The construction of pMT837 is illustrated in fig. 5.

Example 8

Production of chymosin using pMT837.

T. reesei strains QM 9414 and RUT-C-30 were cotransformed with plasmids pMT837 and p3SR2. Plasmid pMT837 contains the prochymosin gene preceeded by the AMG promoter and signal/leader sequence. The construction of plasmid pMT837 was described in Example 7 (see also figs. 3-5). Cotransformation and selection was carried out as in Example 5.

For chymosin production transformants were cultured on minimal medium containing 1 % Solka floc cellulose and 0.2 % proteose peptone.

The mycelia were collected and the supernatant was concentrated, when necessary, by TCA precipitation and diluted into 2 M NaOH 10 mM Tris (pH 8.5). The samples were fractioned on SDS-page (7.5 % - 15 % polyacrylamide gradient) and electroblotted to a nitrocellulose filter. The filters were incubated with rabbit prochymosin antiserum and stained with 4-chloro-1-naftol using α-rabbit-IgG-peroxidase conjugate purchased from Sigma.

Chymosin was shown to be secreted into the medium approximately at the level of 1 µg/l. Since the AMG promoter clearly functions inefficiently in T. reesei homologous T. reesei promoter-terminator vectors were constructed to improve the production level of chymosin.

Example 9

The construction of heterologous expression vectors for production of calf prochymosin in T. reesei using cbh1 promoter.

I. The joining of prochymosin gene to cbh1 terminator region.

The calf prochymosin gene was obtained from plasmid pR27 (fig. 6). The PstI - PstI fragment (~ 1110 bp) containing almost the whole gene was isolated from agarose gel by conventional techniques and ligated to the PstI site of pUC 19.

This plasmid was partially digested with PstI, ends made blunt with S1 nuclease and an AvaII terminator fragment (~ 750 bp) of cbh1 gene (blunt ends with Klenow fragment) was ligated into this plasmid. The terminator region of cbh1 gene was obtained from the terminal 1.8 kb BamHI fragment of cbh1 cDNA subcloned in pBR322 (ref. 24).

This plasmid containing the 'pro C fragment coupled with the terminator region of cbh1 gene in pUC 19 was called pAMH100 (fig. 7).

II. The fusion of cbh1 promoter region and prochymosin coding region using BglI-SacII adaptor.

The Sac II - Pst I fragment (80 bp) coding for the N-terminal region of prochymosin was isolated from plasmid pR 27 (see fig. 6). The cbh1 promoter region was first subcloned from the genomic clone λ 44A as a 2.8 kb long Eco RI - Eco RI fragment into pUC 18 (plasmid pUA 01, fig. 8), and then a ~ 2.2 kb long Eco RI - Bgl I fragment was isolated from this subclone. The Bgl I site is located in the middle of the signal sequence of cbh1 gene. The precise joining of the ~ 2.2 kb long EcoRI - Bgl I fragment, containing the promoter and about half of the signal peptide coding region of cbh1 gene, to the ~ 80 bp Sac II - Pst I prochymosin fragment is mediated by the Bgl I - Sac II adaptor (NOR 202 + NOR 203, fig. 10). These fragments together with the adaptor were ligated into pUC19 digested with Eco RI - Pst I. This plasmid codes for a complete signal sequence of CBH I fused to the first amino acid of prochymosin followed by some of its N-terminal sequences. Finally, from this construction the Eco RI - pcbh1 ss - proC - Pst I

fragment was isolated, ligated from its 3' end into pAMH100 digested with Sal I and Pst I. The 5' end of the fragment and Eco RI-end of plasmid pAMH100 was filled in with Klenow and ligated. Thus the promoter of cbh1 gene and proC' fragment was transferred in front of 'proC followed by the cbh1 terminator area. The construction was named pAMH102 (Fig. 11).

III. The addition of a selectable marker to chymosin expression plasmid.

As a selectable marker in the expression plasmids either the amdS gene of A. nidulans (refs. 42, 30) or the argB gene of A. nidulans can be used (ref. 48)).

The amdS gene was isolated as a Pvu I - Sal I fragment from p3SR2 and ligated to the 6 kb long Pvu I - Sal I fragment of pAMH 102. This selectable vector was named pAMH 104 (Fig. 11).

IV. The precise fusion of the cbh1 promoter and preprochymosin coding sequences using oligonucleotides

The aminoterminal PstI fragment (~150 bp) of preprochymosin was isolated from pR26 (fig. 3) which includes a complete preprochymosin cDNA clone, starting 12 bp upstream from ATG, inserted into PstI site in pBR 322. This fragment was subcloned into the polylinker of pTZ19R together with the cbh1 Eco RI - Sac I (~2.6 kb) promoter fragment which also includes the signal sequence coding region of cbh1 (fig. 8). The cbh1 Eco RI - Sac I fragment is obtained from a 2.8 kb Eco RI - Eco RI subclone in pUC 18 (pUA01, fig. 8) of the original λ44A clone (ref. 24). pTZ19R (Pharmacia) is a pUC19 based plasmid including the F1 origin of replication and the T7 promoter enabling the use of ss-template (single stranded) for oligonucleotide mutagenesis. The construction of the resulting plasmid (pAMH 105) is illustrated in Figure 8. From this plasmid the sequences between cbh1 promoter area and proC ATG were deleted by loop-mutagenesis using a specific oligonucleotide, OAMH 3 (fig. 10). The performance of oligonucleotide directed mutagenesis is illustrated in figure 9. The oligonucleotide in question was phosphorylated, annealed to ss pAMH 105 DNA (ref. 60, ss-DNA was isolated from JM103/pAMH105 as described by Pharmacia) in molar ratio 20:1, respectively. The oligonucleotide primer was elongated using Klenow polymerase and T4 ligase as described in ref. 60. The elongated mixture was digested with Sma I (resides in the polylinker of pTZ19R, see fig. 9) prior to transformation into the mutL mismatch repair deficient

strain of E. coli, BMH71.18 (ref. 61). The pool of transformants was grown overnight in 5 ml liquid culture (Luria broth as described in ref. 62, with 100 µg/ml ampicillin). Plasmid DNA was isolated from pool of transformants and it was redigested with Sma I and retransformed into E. coli JM 109 strain. The screening of potential deletion clones was performed by digestion using different restriction enzymes and the specificity of deletion was further confirmed by sequencing. The resulting plasmid was called pAMH1101 (fig. 12). This plasmid was further digested with Eco RI and Pst I and the resulting pcbh1-preproC fragment was isolated (fig. 14) and ligated at its PstI-end to pAMH100 plasmid digested with PstI and SalI. The ends of the resulting ligated fragment were made blunt by Klenow and ligated. The resulting plasmid was called pAMH101 and it contains the cbh1 promoter area fused to the signal sequence of prochymosin and the whole prochymosin coding region fused to the terminator area of cbh1 gene (fig. 14).

V. The precise fusion of the cbh1 promoter and preproC gene by signal sequence fusion performed by using oligonucleotides.

The construction of plasmid pAMH1103 (fig. 12) was performed essentially as pAMH1101 described in details in the preceding chapter IV with the exception that the specific oligonucleotide used for this construction was OAMH1 (fig. 10 and 9). The resulting plasmid pAMH 1103 contains a signal sequence fusion including amino acids (aa) 1-12 from cbh1 signal sequence fused to aa 12-16 from preproC signal sequence preceding the aminoterminal part (~ 140 bp) of the coding region of proC gene (fig. 12). From plasmid pAMH 1103 the pcbh1-sso-proC' (o = fusion) fragment was subcloned into pAMH 100 essentially as described in the preceding chapter IV (see Fig. 12). The resulting plasmid pAMH 103 includes the promoter area of cbh1 and signal sequence fusion of cbh1 and preproC preceding the proC coding region fused to the cbh1 terminator area.

VI The precise fusion of the cbh1 coding region to proC coding region by using oligonucleotides.

The construction of plasmid pAMH 1106 (fig. 13) was performed essentially as pAMH 1101 described in details in the preceding chapter IV with the exception that the specific oligonucleotide used for this construction was OAMH2 (fig. 10 and 9). From the resulting plasmid pAMH 1106 including the promoter area of cbh1, signal sequence of cbh1 and the coding

region of 1-20 first aa of mature CBH I fused to the coding region of proC the fragment containing pcbh1-mature o-proC' was subcloned into pAMH100 essentially as described in the preceding chapter IV (Fig. 12). The resulting plasmid pAMH106 includes the promoter area of cbh1, signal sequence of cbh1, coding region of aa 1-20 of mature CBHI fused to the coding region of proC.

Example 10

Production of chymosin using pAMH102 and pAMH 104.

T. reesei strains QM 9414 and RUT-C-30 were cotransformed with plasmids pAMH102 and p3SR2 in molar ratio 5:1, respectively. The construction of plasmid pAMH102 was described in Example 9 chapter II. Cotransformation and selection was carried out as in Example 5. The transformants were purified on selective asetamide plates as described in Example 3.

For chymosin production transformants were cultured on minimal medium (10 to 50 ml) containing 1 % Solka floc cellulose and 0.2 % proteose peptone. The mycelia were collected and the supernatant was concentrated by TCA precipitation and diluted into 2M NaOH 10 mM Tris (pH 8.5). The mycelia were broken in the presence of liquid nitrogen, broken cells were pelleted and the supernatant treated in the same way as the culture media. The samples were fractioned on SDS-page (7.5 % - 15 % polyacrylamide gradient) and electroblotted to a nitrocellulose filter. The filters were incubated with rabbit prochymosin antiserum and α-rabbit-IgG-AP (alkaline phosphatase) conjugate and stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate purchased from Promega Biotec. The amount of chymosin inside and outside the mycelium was compared (fig. 17). It was shown by Southern hybridization that the clones containing higher number of copies of plasmid pAMH102 integrated into the fungal chromosomal DNA also produced more chymosin. When using a one copy transformant the amount of secreted chymosin was 100 µg/l of culture medium when approximated from Western gels (fig. 17). The secreted prochymosin was shown to be processed to an active chymosin by Western gels and by the milk clotting activity (chymosin clots milk by cleavage of β-kasein determination (ref. 63). The amount of various forms of chymosin (preproC, proC, C and chymosin derived degradation products inside the cell) inside the mycelium was determined to be 0.04 % of mycelial total protein (fig. 17) and the amount of secreted chymosin was 60 % of the total chymosin produced. This shows the efficiency of T. reesei to secrete even heterologous gene products outside the mycelia.

Transformants with several copies of plasmid pAMH104 (fig. 11), able to secrete larger amounts of prochymosin were screened for by determining the milk clotting activity of the growth media. The best transformant out of the 40 studied was found to secrete 500 µg/l of culture medium as determined on Western gels and by milk clotting activity.

Since the amount of secreted, active chymosin was increased 200-fold when cultures were grown in a 5 l laboratory fermenter compared to the small-scale cultures (10-50 ml), the amount of chymosin produced by this type of strain is around 100 mg/l.

Example 11

Construction of a general expression vector for production of homologous and heterologous proteins in T. reesei.

In order to be able to construct vectors for production of various proteins in T. reesei a general expression vector including the promoter and terminator areas of cbh1 gene was constructed. The cbh1 terminator was subcloned together with an adaptor including the STOP codon TAA in three reading frames into Pst I site of pUC19 resulting in plasmid pAMH110' (fig. 15). The Pst I terminator fragment was isolated from pAMH110', the cbh1 promoter area was isolated from pAMH102 as an Eco RI - Pst I fragment including the aminoterminus of proC gene and these fragments were subcloned into an Eco RI - Pst I digested pUC19* from which the single Nde I site had been made blunt with Klenow prior to this subcloning step. The resulting plasmid pAMH110 includes the promoter and terminator of cbh1 gene and between these sequences a stuffer fragment which can be removed by digestion with Sac II and Nde I. After the ends are made blunt any cDNAs or chromosomal copies of genes can be inserted between the promoter and terminator (fig. 15).

Example 12

The expression of T. reesei endoglucanase I under cbh1 promoter.

In order to increase the amount of endoglucanase produced, the egl1 gene was linked to the more powerfull cbh1 promoter. The cDNA for T. reesei endoglucanase I was subcloned as an Eco RI - Bam HI fragment into general expression vector pAMH110 (described in example 11) which was first digested with Sac II - Nde I to delete the stuffer fragment (fig. 16). The resulting plasmid pAMH111 including egl1 gene between the promoter and terminator of cbh1 gene was cotransformed with p3SR2 to T. reesei QM 9414 (ATCC 26921) in

molar ratio 5:1, respectively. The transformants were selected for AmdS$^+$ phenotype and further purified on selective medium. Six individual transformants were grown for 4 days in cellulase inducing medium (Solka floc as a carbon source, 1 %) in 50 ml liquid cultures. The culture supernatants were then tested for endoglucanase activity by measuring the release of reducing sugars from hydroxyethylcellulose (0.1 %, ref. 12). The EG I activities of transformants were compared to a control (QM 9414) and the best transformant was shown to secrete 4 times the endoglucanase activity of the control strain. This example shows that it is possible to modify the amounts of different cellulolytic enzymes in T. reesei by changing the respective promoter.

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60. Marsh, J.L., Erfle, M. & Wykes, E.J. The pIC plasmid and phage vectors with verstile cloning sites for recombinant selection by insertional inactivation. Gene 32 (1984), 481-485.

61. Vieira, J. & Messing, J., The pUC plasmids, an M13mp7 - derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19 (1982), 259-268.

62. Van Gorcom, R.F.M., Pouwels, P.H., Goosen, T., Visser, J., Van den Broek, H.W.J., Hamer, J.E., Timberlake, W.E. & Van den Hondel, C.A.M.J.J. Expression of an Escherichia coli β-galactosidase fusion gene in Aspergillus nidulans. Gene 40 (1985) 99-106.

63. Nummi, M., Niku-Paavola, M.-L., Lappalainen, A., Enari, T.-M. & Raunio, V., Cellobiohydrolase from Trichoderma reesei. Biochem.J. 215 (1983), 677-683.

64. Eghtedarzadch, M.K. & Henikoff, S., Use of oligonucleotides to generate large deletions. Nucl. Acids Res. 14 (1986), 5115.

65. Obtained from Paul Thomas, Imperial College, Dept. of Chemistry, London.

66. Miller, J.H., Experiments in molecular genetics (1972), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

67. The method for determining the activity of chymosin in cleavage of milk β-kasein has been described by Siika-aho, M. (1983) Mikrobirenniinin tuottaminen, MSc Thesis. Biotechnical Laboratory, VTT, Tietotie 2, 02150 Espoo, Finland.

The features disclosed in the foregoing description, in the following claims and/or in the accompanying drawings may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

CLAIMS

1. A vector system for use in the transformation of Trichoderma comprising

a) a gene encoding a desired protein product,

b) functions facilitating gene expression including promoters/enhancers operably linked to control expression of the desired product, and

c) a selection marker.

2. A vector system according to claim 1 further comprising a signal/leader sequence fused upstream to the 5'end of the gene for the desired protein product.

3. A vector system according to claim 1 or 2, wherein the promoter can function in Trichoderma.

4. A vector system according to claim 1, wherein the promoter is derived from a gene for protein heterologous to Trichoderma.

5. A vector system according to claim 2, wherein the signal/leader sequence is derived from a gene for a protein secreted by Trichoderma.

6. A vector system according to claim 2, wherein the signal/leader sequence is derived from a secreted protein heterologous to Trichoderma.

7. A vector system according to claim 5 to 6, wherein the signal/leader sequence is derived from the signal/leader sequence of the desired proteins.

8. A vector system according to claim 5, wherein the signal sequence is the Trichoderma reesei cbhl signal sequence.

9. A vector system according to claim 6, wherein the signal sequence is the Aspergillus glucoamylase signal sequence.

10. A vector system according to claim 1, wherein the desired protein is homologous to Trichoderma.

11. A vector system according to claim 1, wherein the desired protein is heterologous to Trichoderma.

12. A vector system according to claim 11, wherein the desired product is prochymosin.

13. A vector system according to claim 10, wherein the desired protein is T. reesei endoglucanase (EG I).

14. A vector system according to claim 3, wherein the promoter is the Trichoderma reesei cbhl promoter.

15. A vector system according to claim 4, wherein the promoter is the Aspergillus glucoamylase promoter.

16. A vector system according to claim 1, wherein the selection marker is derived from the Aspergillus nidulans amdS gene, the Aspergillus nidulans or Trichoderma reesei argB gene or the Neurospora crassa or Trichoderma reesei pyr4 gene.

17. A vector system according to claim 1 comprising at least two plasmids or vectors.

18. A vector system according to claim 17, wherein the selection marker is situated on one plasmid and the remaining DNA-sequences to be incorporated in the host genome are situated on another plasmid.

19. Trichoderma strains stably transformed with a vector system in accordance with any of the previous claims 1-18.

20. A method for transformation of Trichoderma, wherein a suitable Trichoderma strain is transformed with a vector system in accordance with any of the previous claims 1-18.

21. A method for the production of a protein product in Trichoderma, wherein a suitable host microorganism is transformed with a vector system in accordance with any one of claims 1-18, the transformed strain is cultured in a suitable culture medium and the expressed and secreted protein product is recovered from the culture medium.

22. A method for the production of a protein product in Trichoderma which comprises cultivating in a suitable culture medium a suitable host microorganism transformed with a vector system in accordance with any one of claims 1-18 and recovering the expressed and secreted protein product from the culture medium.

23. A method in accordance with claims 21 or 22, wherein the host microorganism is a prototrophic T. reesei strain.

24. A method in accordance with claim 21 or 22, wherein the host microorganism is an auxotrophic T. reesei strain.

25. A method in accordance with anyone of claims 21-24, wherein the host strain is deficient to any gene encoding an undesired product.

26. A method according to claim 25, wherein the host strain is deficient in the cbhl gene.

27. Auxotrophic Trichoderma strains usable as host strains to be transformed with the vector system in accordance with any of claims 1-18.

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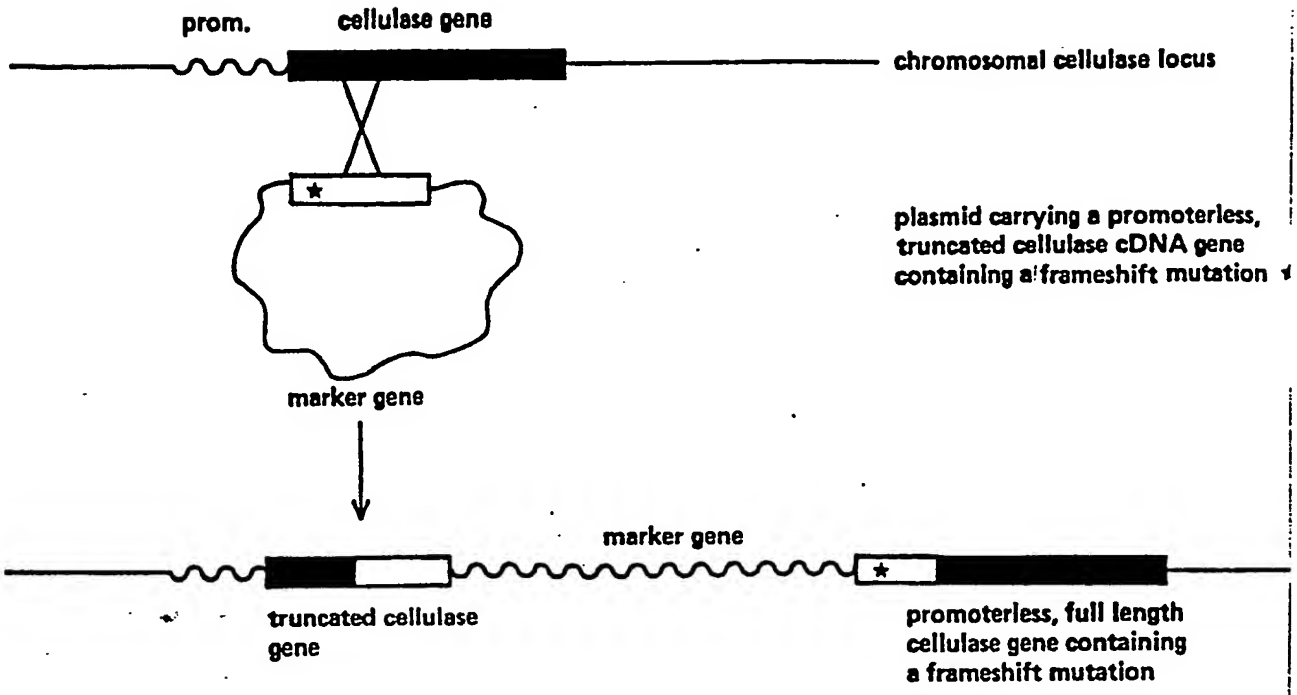
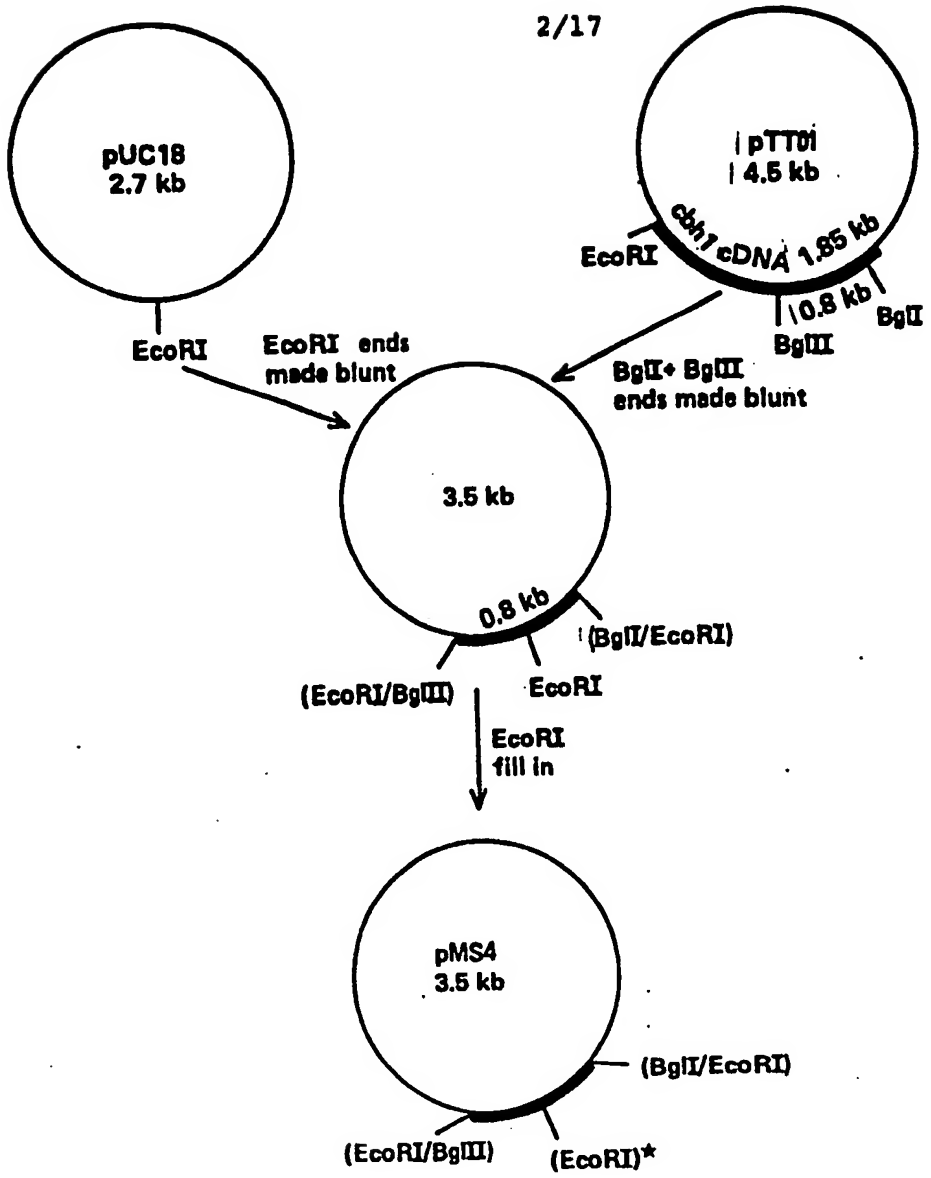


Fig. 1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2.7 kb
EcoRI
EcoRI ends
made blunt
pTT01
4.5 kb
cbh1 cDNA 1.85 kb
EcoRI
0.8 kb
BglII
BglIII
BglII+ BglIII
ends made blunt
3.5 kb
0.8 kb
(BglII/EcoRI)
(EcoRI/BglIII)
EcoRI
EcoRI
fill in
pMS4
3.5 kb
(BglII/EcoRI)
(EcoRI/BglIII)
(EcoRI)*

Fig. 2

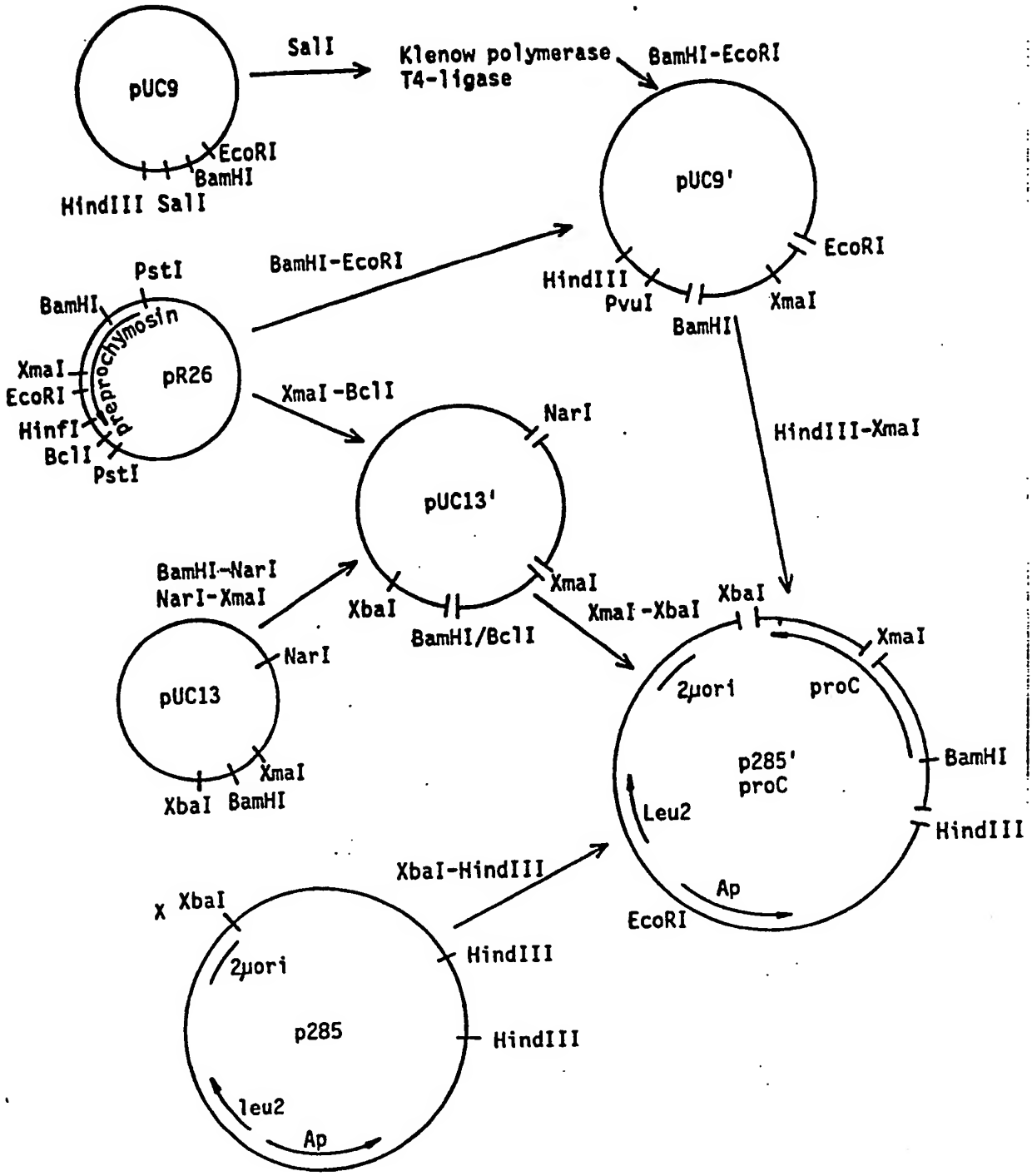
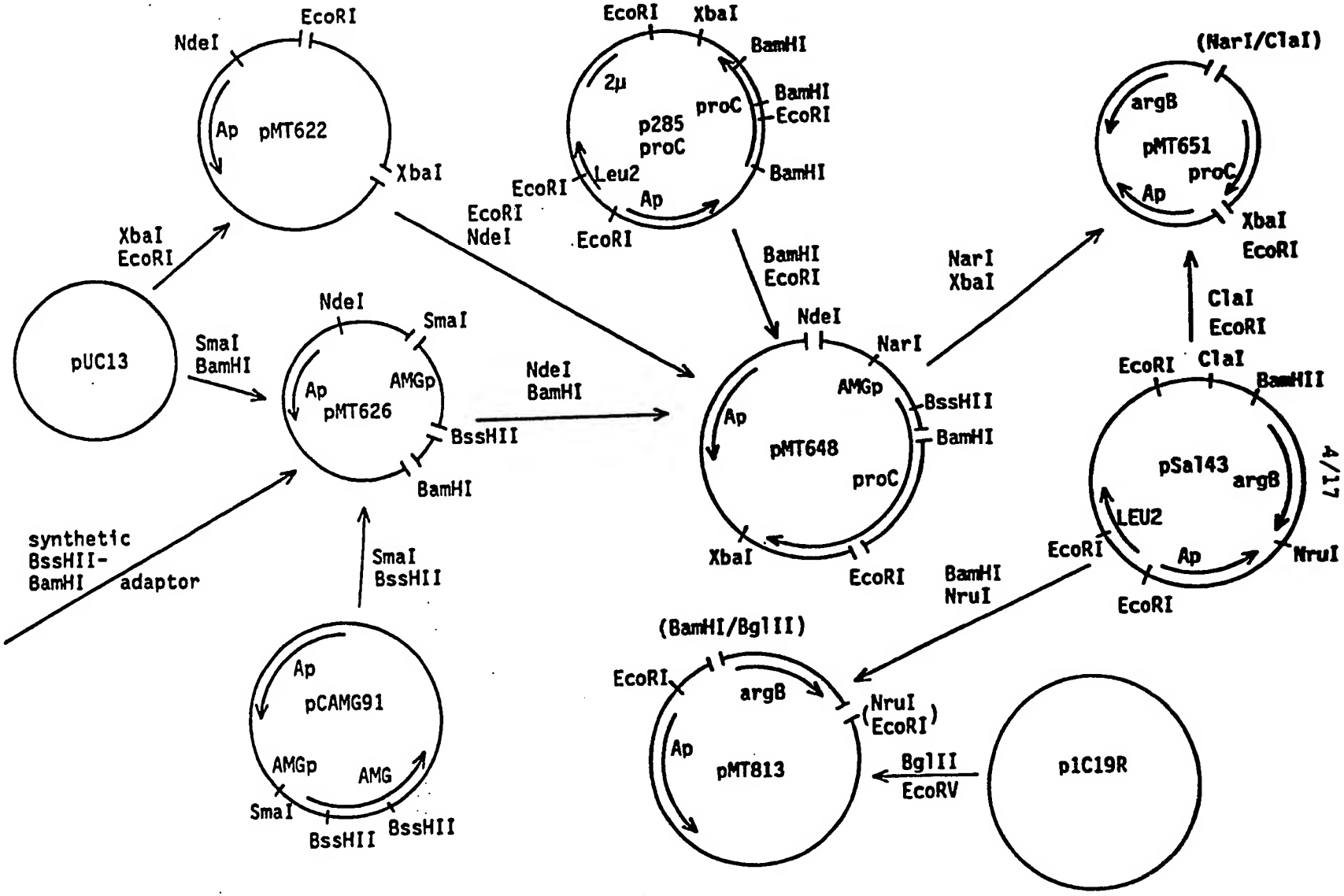

pUC9
SalI
Klenow polymerase
T4-ligase
BamHI-EcoRI
EcoRI
BamHI
HindIII SalI
pUC9'
EcoRI
HindIII
PvuI
XmaI
BamHI
BamHI-EcoRI
PstI
BamHI
XmaI
EcoRI
HinfI
BclI
PstI
pR26
preprochymosin
XmaI-BclI
NarI
pUC13'
XmaI
XbaI
BamHI/BclI
HindIII-XmaI
BamHI-NarI
NarI-XmaI
pUC13
NarI
XmaI
XbaI
BamHI
XmaI-XbaI
XbaI
XmaI
2μori
proC
p285'
proC
BamHI
Leu2
HindIII
Ap
EcoRI
XbaI-HindIII
X XbaI
2μori
HindIII
p285
HindIII
leu2
Ap

Fig. 3


EcoRI
NdeI
Ap
pMT622
XbaI
XbaI
EcoRI
pUC13
SmaI
BamHI
NdeI
SmaI
Ap
AMGp
pMT626
BssHII
BamHI
synthetic
BssHII-
BamHI
adaptor
SmaI
BssHII
Ap
pCAMG91
AMGp
AMG
SmaI
BssHII
BssHII
EcoRI
XbaI
BamHI
2μ
p285
proC
BamHI
EcoRI
Leu2
Ap
EcoRI
EcoRI
NdeI
EcoRI
BamHI
EcoRI
NdeI
NarI
Ap
AMGp
BssHII
pMT648
BamHI
proC
XbaI
EcoRI
NarI
XbaI
(NarI/ClaI)
argB
pMT651
proC
Ap
XbaI
EcoRI
ClaI
EcoRI
EcoRI
ClaI
BamHII
pSal43
argB
LEU2
Ap
NruI
EcoRI
EcoRI
4/17
BamHI
NruI
(BamHI/BglII)
EcoRI
argB
Ap
pMT813
NruI
(EcoRI)
BglII
EcoRV
p1C19R

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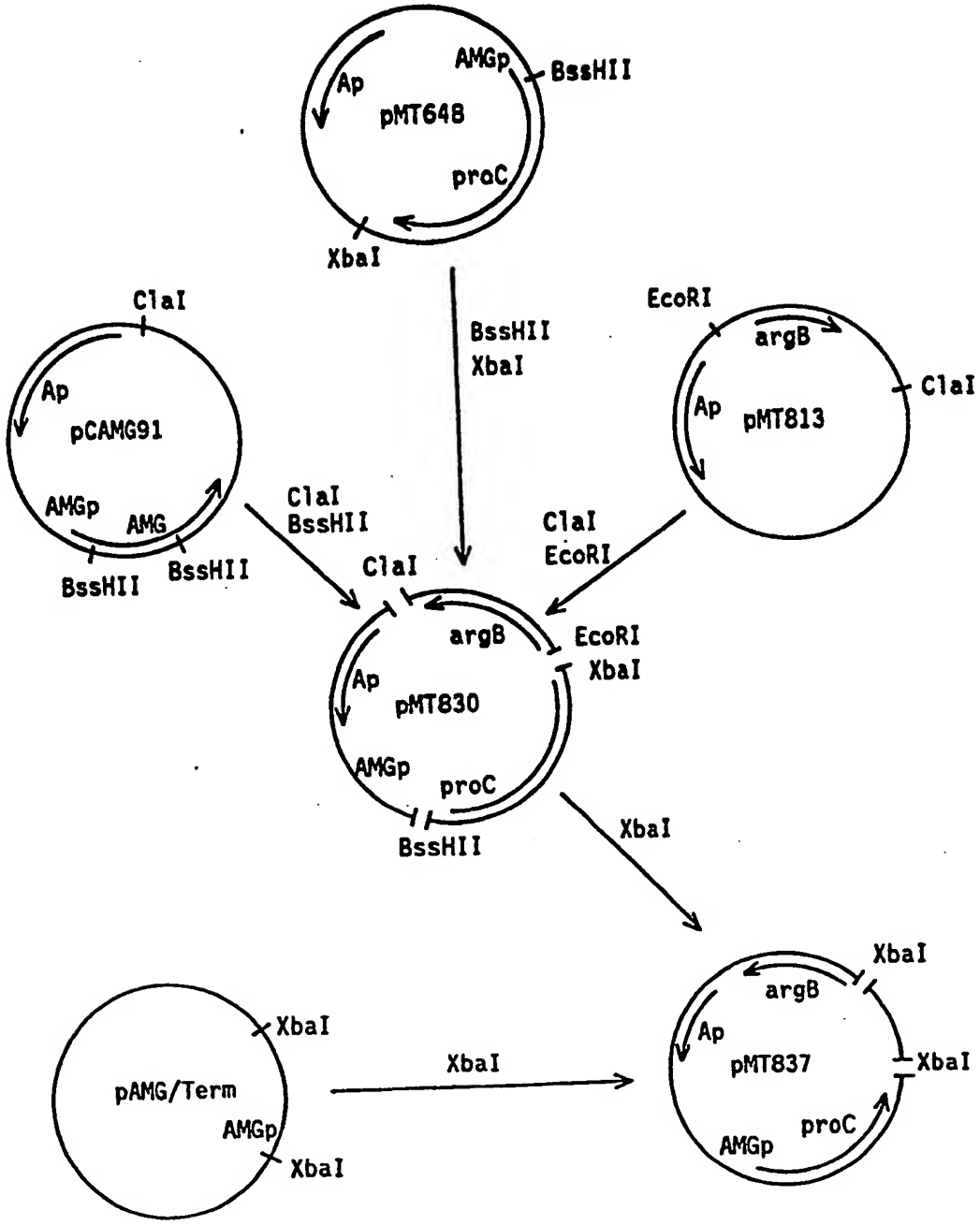
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Ap
pMT648
proC
XbaI
ClaI
BssHII
XbaI
EcoRI
argB
Ap
pMT813
ClaI
ClaI
Ap
pCAMG91
AMGp
AMG
BssHII
BssHII
ClaI
BssHII
ClaI
EcoRI
ClaI
argB
EcoRI
Ap
pMT830
XbaI
AMGp
proC
BssHII
XbaI
XbaI
pAMG/Term
AMGp
XbaI
XbaI
XbaI
argB
Ap
pMT837
XbaI
AMGp
proC

Fig. 5

The N-terminal linker of pR27: PstI/PvuII/BamHI - SacII/XmaIII

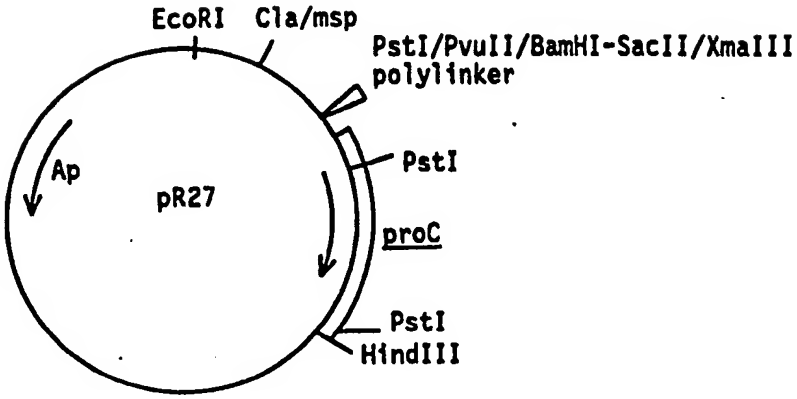
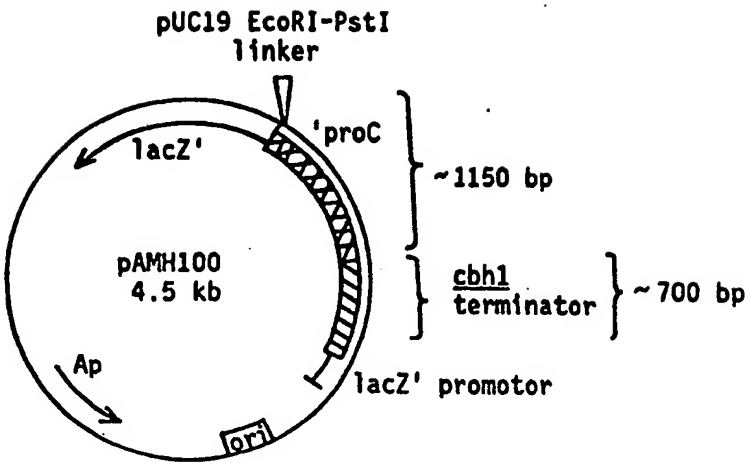


Fig. 6



pAMH100: 'proC - cbh1 terminator construction

Fig. 7

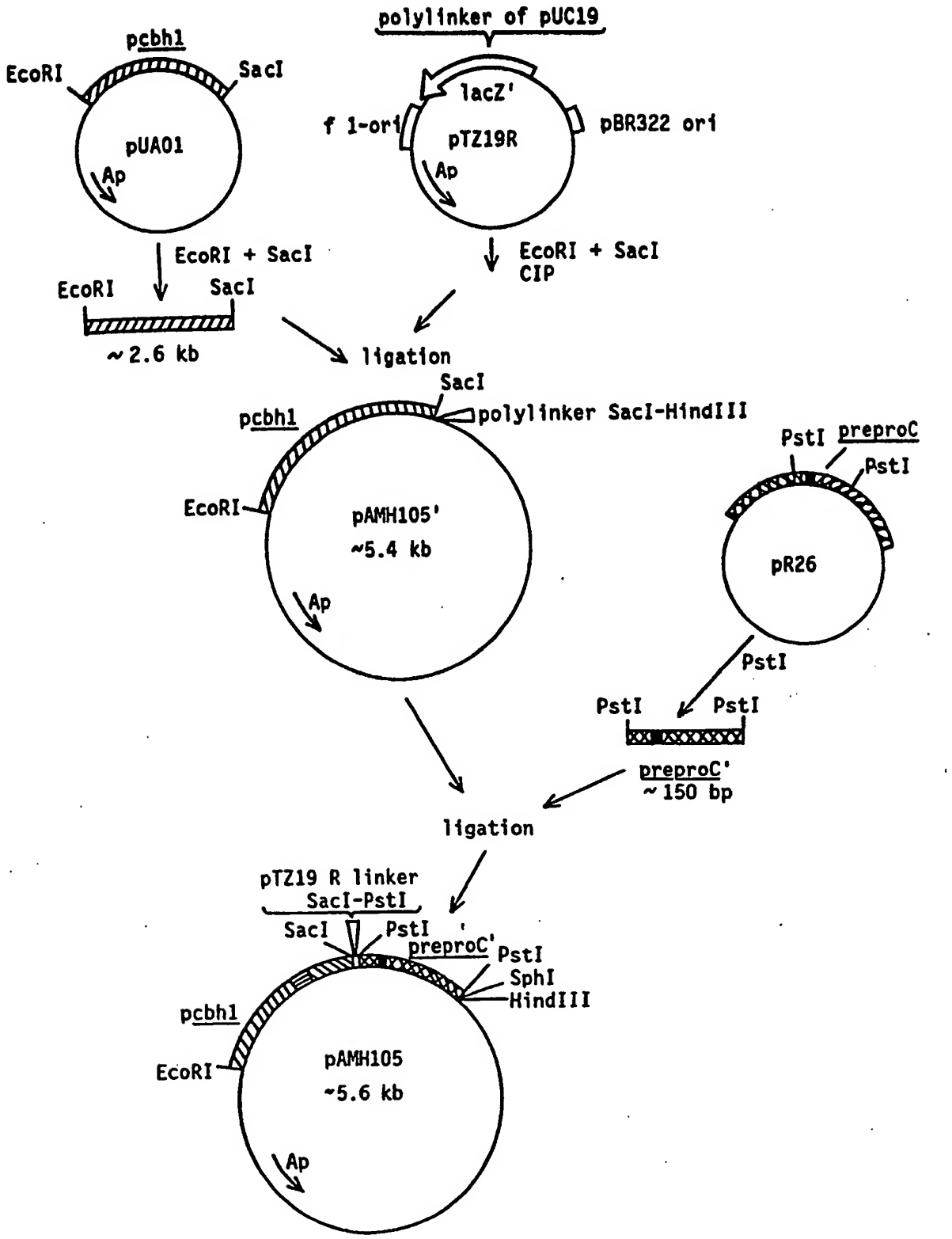
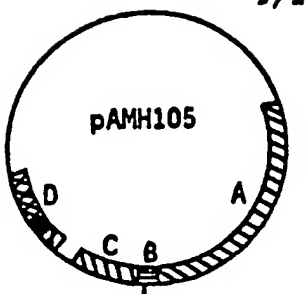

polylinker of pUC19
pcbh1
EcoRI
SacI
pUA01
Ap
lacZ'
f 1-ori
pTZ19R
pBR322 ori
Ap
EcoRI + SacI
EcoRI + SacI
CIP
EcoRI
SacI
~2.6 kb
ligation
SacI
pcbh1
polylinker SacI-HindIII
EcoRI
pAMH105'
~5.4 kb
Ap
PstI
preproC
PstI
pR26
PstI
PstI
PstI
preproC'
~150 bp
ligation
pTZ19 R linker
SacI-PstI
SacI
PstI
preproC'
PstI
SphI
HindIII
pcbh1
EcoRI
pAMH105
~5.6 kb
Ap

Fig. 8



A = pcbh1 area (~2.4 kb)

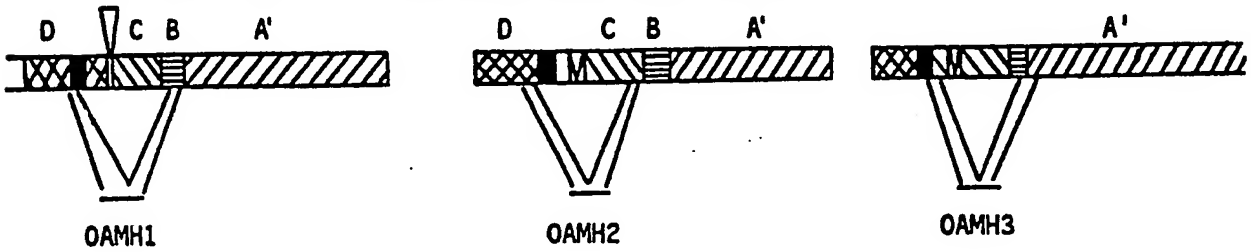
B = ss of cbh1

C = 5' end of the cbh1 gene coding for the amino acids 1-57

D = a 140 bp fragment from pR26 including the preproC 5' end until the first PstI site in the coding region of the gene, ■ =ss

Annealing of one of the synthetic oligonucleotides OAMH1, OAMH2 or OAMH3 (Fig. 10).

polylinker of pTZ19R
including SacI-KpnI-XmaI/SmaI-BamHI-XbaI-SalI/AccI/HincII-PstI



OAMH1 OAMH2 OAMH3

- elongation with Klenow and ligase
- digestion with SmaI (SmaI in the polylinker)
- transformation I
- isolation of plasmid DNA from the pool of transformants
- digestion with SmaI
- transformation II
- the screening of right clones
- sequencing of the potential right clones

pAMH1103 pAMH1106 pAMH1101

Fig. 9

NOR 202

5' TG'GCC'ACA'GCT'CGT'GC 3'

NOR 203

5' ACG'AGC'TGT'GGC'CAA'GA 3'

oligo for making a ss fusion (Φ)

OAMH1 3' TAG'AGC'CGG'AAG'AAC'CGA'GAG'AGG'GTC'CCG' 5'

coding region of aa 8-12 from CBHI ss ← → coding region of aa 12-16 from proC ss

oligo for making a fusion between mature proteins

OAMH2 3' ACC'GTC'TTT'ACG'AGC'CCG'CGA'CTC'TAG'TGG' 5'

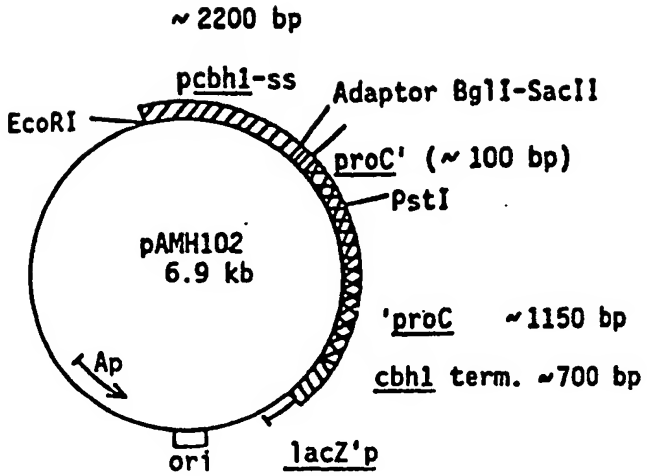
coding region of aa 16-20 from mature CBHI ← → coding region of aa 16-20 from preproC

oligo for joining the promoter of cbh1 to chymosin ss

OAMH3 3' GCC'TGA'CCG'TAG'TAC'TCC'ACA'GAG'CAC' 5'

12 ATG preceding bases from cbh1 gene ← → coding region of 5 first aa from preproC

Fig. 10


~2200 bp
pcbh1-ss
Adaptor BglI-SacII
EcoRI
proC' (~100 bp)
PstI
pAMH102
6.9 kb
'proC ~1150 bp
Ap
cbh1 term. ~700 bp
ori
lacZ'p

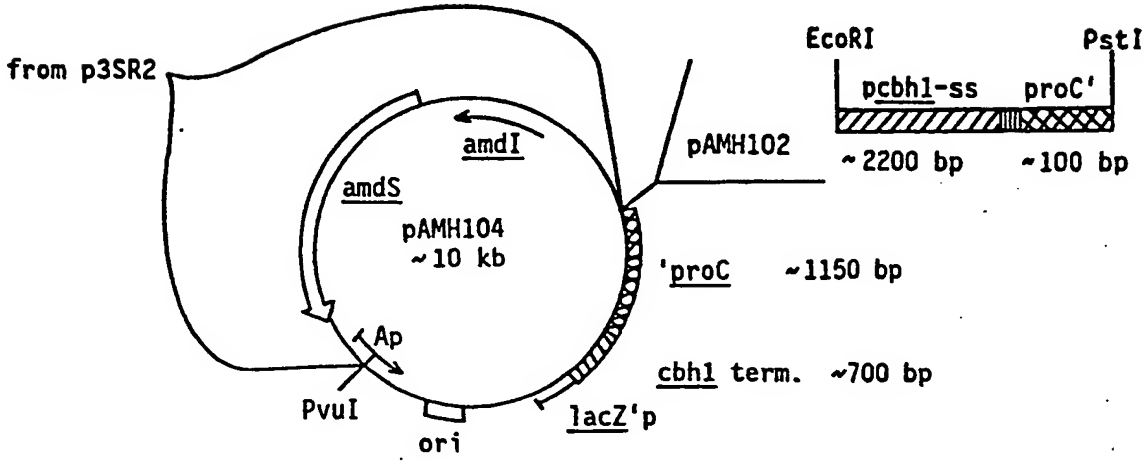

from p3SR2
EcoRI
PstI
pcbh1-ss
proC'
amdI
pAMH102
~2200 bp
~100 bp
amdS
pAMH104
~10 kb
'proC ~1150 bp
Ap
cbh1 term. ~700 bp
PvuI
lacZ'p
ori

Fig. 11

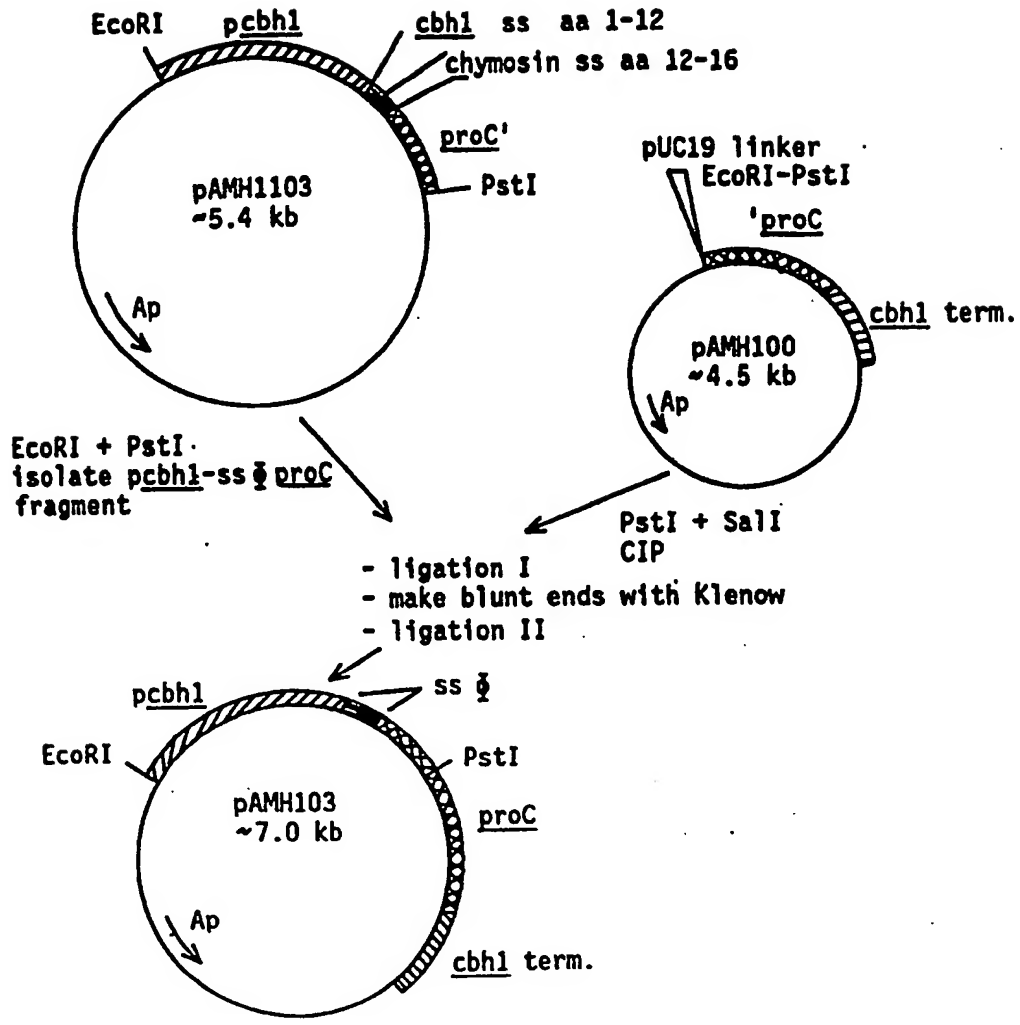

EcoRI
pcbh1
cbh1 ss aa 1-12
chymosin ss aa 12-16
proC'
PstI
pAMH1103
~5.4 kb
Ap
pUC19 linker
EcoRI-PstI
'proC
cbh1 term.
pAMH100
~4.5 kb
Ap
EcoRI + PstI
isolate pcbh1-ss Φ proC
fragment
PstI + SalI
CIP
- ligation I
- make blunt ends with Klenow
- ligation II
pcbh1
ss Φ
EcoRI
PstI
pAMH103
~7.0 kb
proC
Ap
cbh1 term.

Fig. 12

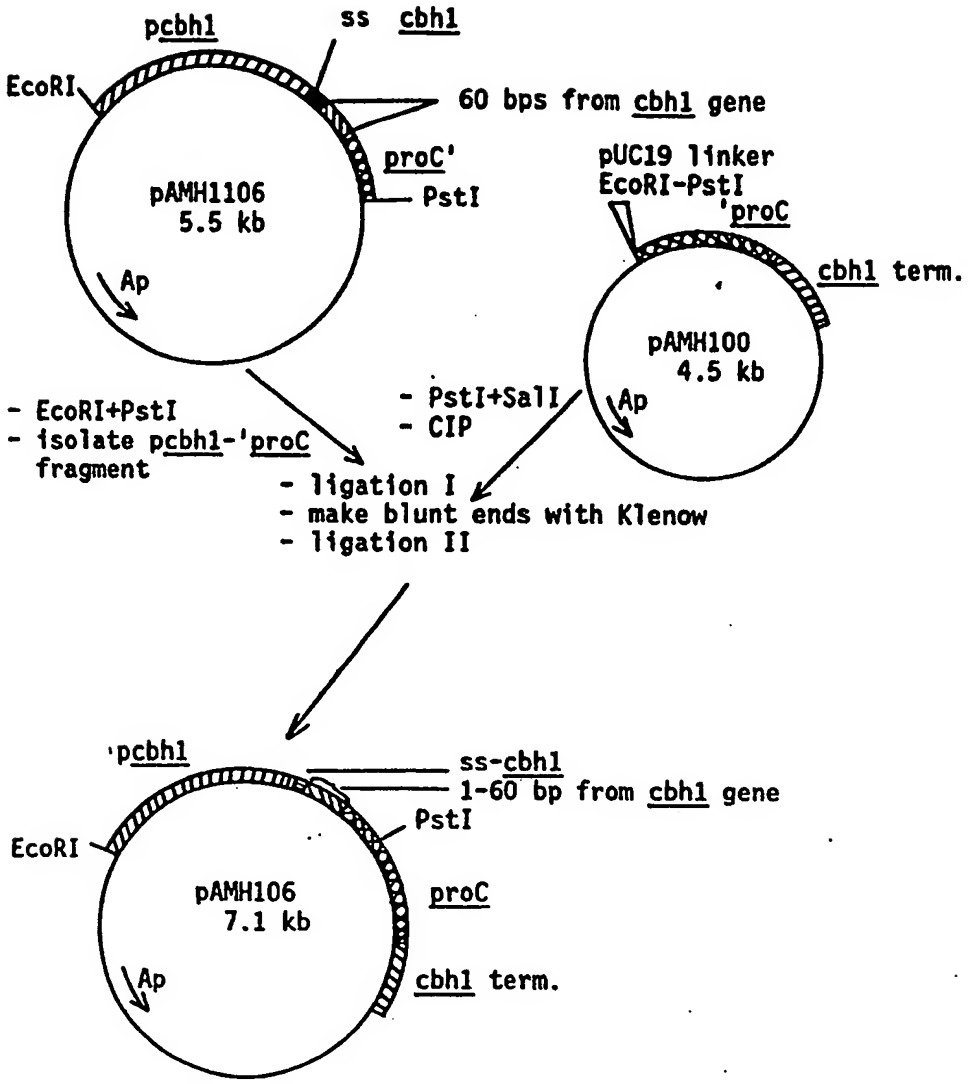

pcbh1
ss cbh1
EcoRI
60 bps from cbh1 gene
proC'
pAMH1106
5.5 kb
PstI
Ap
pUC19 linker
EcoRI-PstI
'proC
cbh1 term.
pAMH100
4.5 kb
Ap
- EcoRI+PstI
- isolate pcbh1-'proC fragment
- PstI+SalI
- CIP
- ligation I
- make blunt ends with Klenow
- ligation II
'pcbh1
ss-cbh1
1-60 bp from cbh1 gene
PstI
EcoRI
pAMH106
7.1 kb
proC
Ap
cbh1 term.

Fig. 13

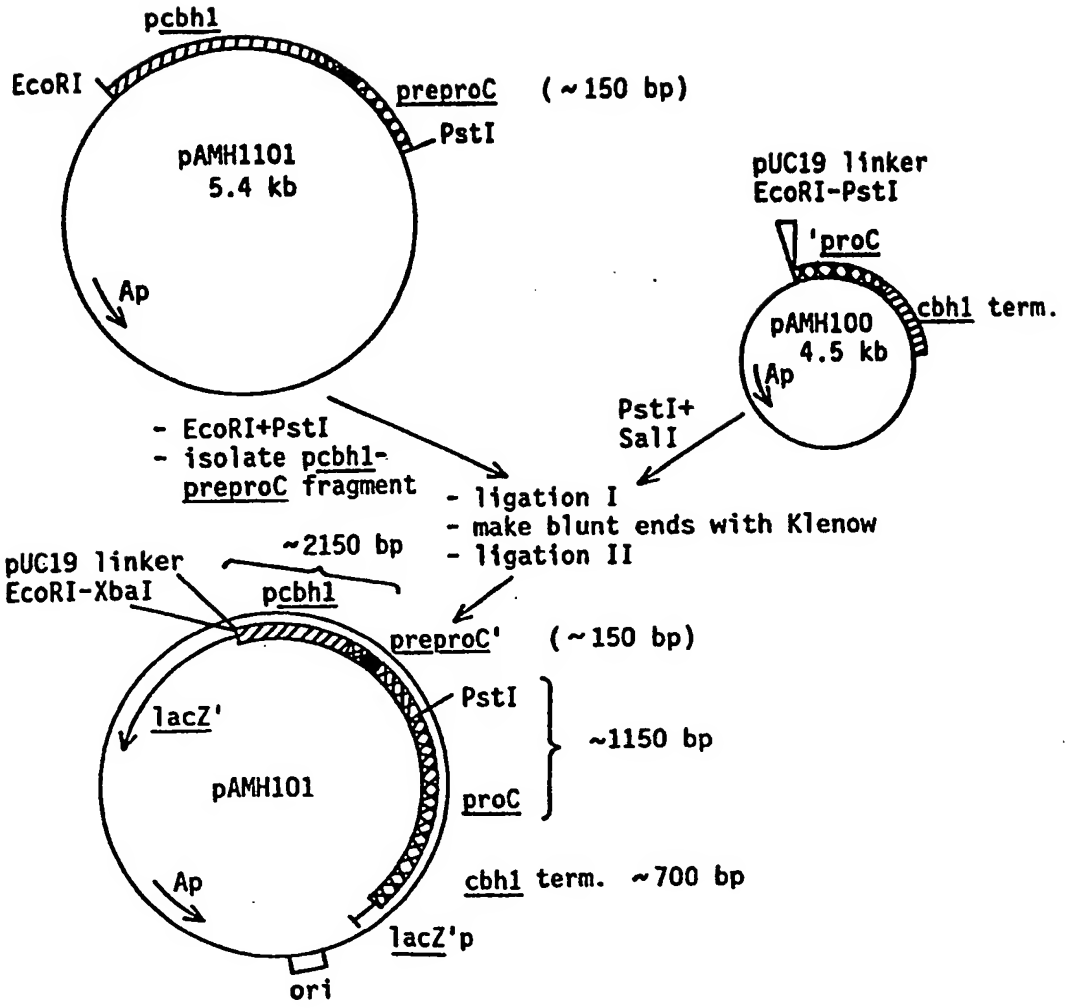

pcbh1
EcoRI
preproC (~150 bp)
PstI
pAMH1101
5.4 kb
Ap
pUC19 linker
EcoRI-PstI
'proC
cbh1 term.
pAMH100
4.5 kb
Ap
PstI+
SalI
- EcoRI+PstI
- isolate pcbh1-preproC fragment
- ligation I
- make blunt ends with Klenow
- ligation II
~2150 bp
pUC19 linker
EcoRI-XbaI
pcbh1
preproC' (~150 bp)
PstI
~1150 bp
lacZ'
pAMH101
proC
Ap
cbh1 term. ~700 bp
lacZ'p
ori

Fig. 14

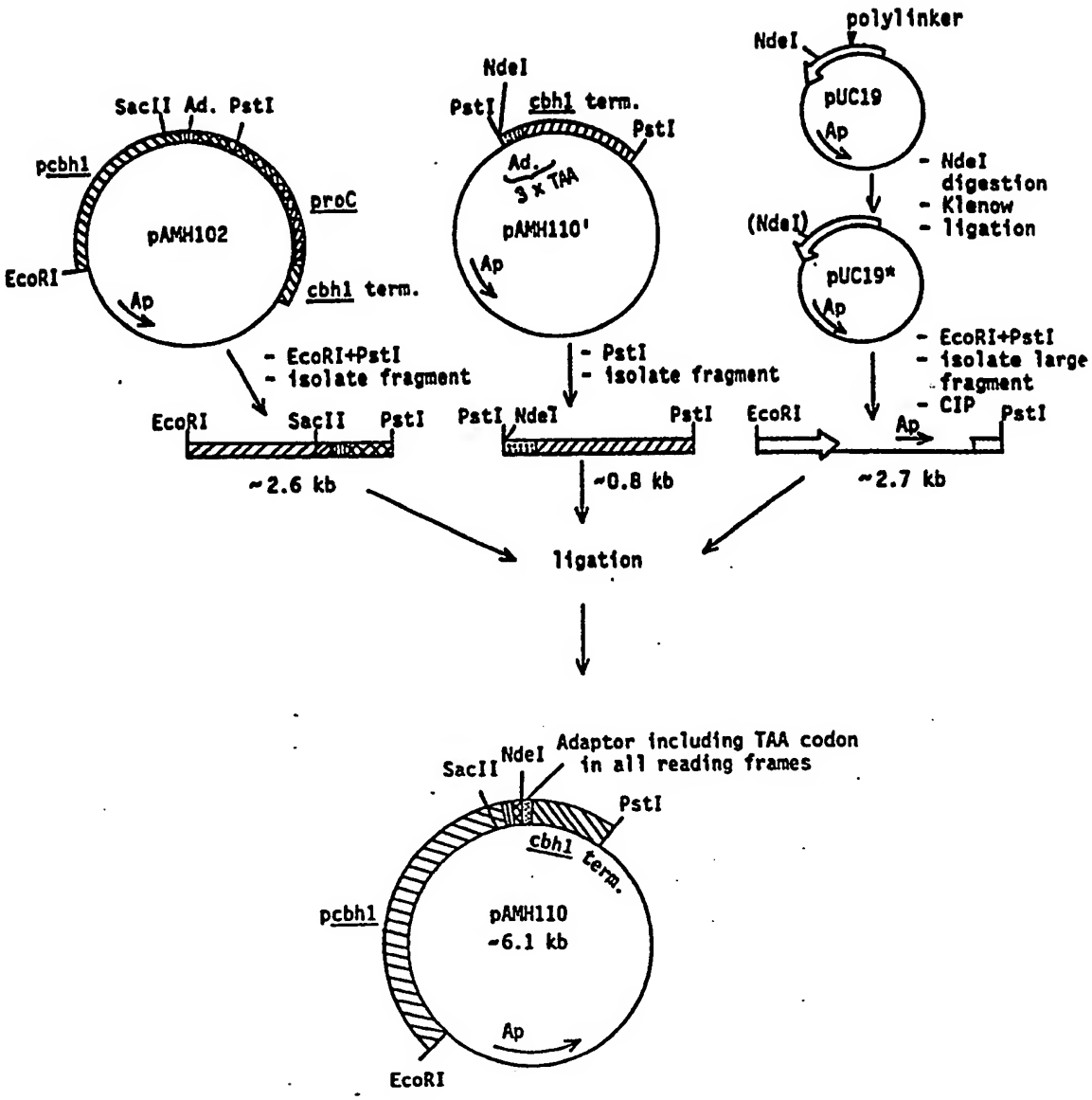

SacII Ad. PstI
pcbh1
proC
pAMH102
EcoRI
Ap
cbh1 term.
- EcoRI+PstI
- isolate fragment
EcoRI SacII PstI
~2.6 kb
NdeI
PstI cbh1 term.
PstI
Ad.
3 x TAA
pAMH110'
Ap
- PstI
- isolate fragment
PstI NdeI PstI
~0.8 kb
polylinker
NdeI
pUC19
Ap
- NdeI digestion
- Klenow
- ligation
(NdeI)
pUC19*
Ap
- EcoRI+PstI
- isolate large fragment
- CIP
EcoRI
Ap
PstI
~2.7 kb
ligation
Adaptor including TAA codon in all reading frames
SacII
NdeI
PstI
cbh1 term.
pcbh1
pAMH110
~6.1 kb
Ap
EcoRI

Fig. 15

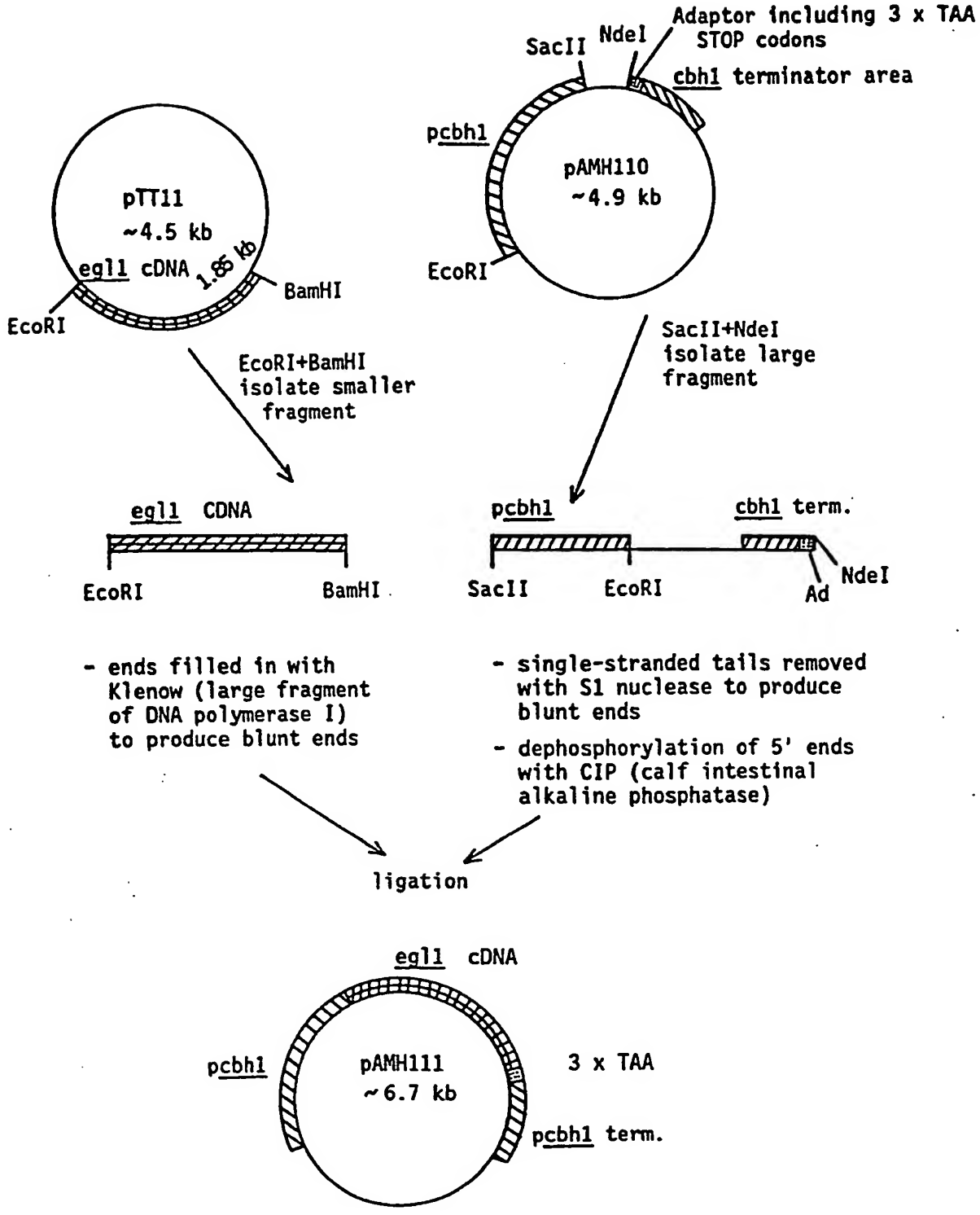

Adaptor including 3 x TAA STOP codons
SacII
NdeI
cbh1 terminator area
pcbh1
pAMH110
~4.9 kb
EcoRI
pTT11
~4.5 kb
egl1 cDNA
1.85 kb
BamHI
EcoRI
EcoRI+BamHI isolate smaller fragment
SacII+NdeI isolate large fragment
egl1 CDNA
EcoRI
BamHI
pcbh1
cbh1 term.
SacII
EcoRI
Ad
NdeI
- ends filled in with Klenow (large fragment of DNA polymerase I) to produce blunt ends
- single-stranded tails removed with S1 nuclease to produce blunt ends
- dephosphorylation of 5' ends with CIP (calf intestinal alkaline phosphatase)
ligation
egl1 cDNA
pcbh1
pAMH111
~6.7 kb
3 x TAA
pcbh1 term.

Fig. 16

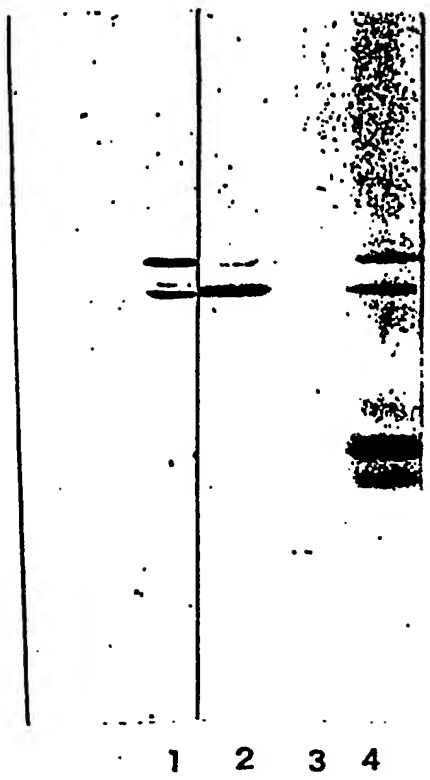


Fig. 17